Methylene Blue Active Substances (MBAS) assay for nonionic surfactants in environmental samples.
Method Version 081408.

Developed by Jack Bell, Ph. D., with assistance from Geneva Mottet and Hannah Halliday, under the direction of Russel Barsh, Kwiaht, and using the facilities of the University of Washington Friday Harbor Marine Laboratories. This procedure is written for a student new to analytical chemistry.

Principle of the method:
Water-soluble anionic surfactants such as linear alkyl sulfates form a 1:1 ion pair with the water-soluble cationic dye, methylene blue (MB). The ion pair is effectively neutral and is therefore extractable into a water-insoluble organic solvent such as dichloromethane (DCM). The blue complex is measured at 650 nm.

The limit of detection (LOD) for this version of the method is approximately 0.1 parts-per-million (ppm). The LOD is governed by the relative and total volumes of the upper aqueous phase and the lower DCM organic phase. The current method uses a 2 mL aqueous phase and a 2 mL DCM phase. This is an aqueous:organic volume ratio of 1:1 and a small total volume for minimum bench space, glassware and solvent usage, appropriate for public demonstrations and instructional uses. The standard EPA-approved method uses aqueous:organic phase ratios as high as 200:1 to attain LOD's in the parts-per-billion (ppb) range. Large separatory funnels and more tedious procedures are required.

Interferences: Any chemical in the sample with an anionic group ($-SO_3^-$, $-OSO_3^-$, $-CO_2^-$) and a hydrocarbon portion (C_nH_n) will interfere, that is, cause a false-positive result. Nonionic surfactants do not interfere. "Nonionics" are measured in the Cobalt Thiocyanate Active Substances (CTAS) assay.

Equipment and labware:
1. Double beam spectrophotometer, zeroed on air at 650 nm. Allow warm up until absorbance reading is stable, usually about 15 minutes. Read all

samples in a 1.5 mL glass cuvette versus air in the reference beam (empty rear cuvette holder). The sample compartment lid is partly open due to the height of the test tubes. Under stable fluorescent lighting and in the absence of transient shadows, readings are stable.
2. Glass test tubes (13 X 100 mm) with screw caps lined with DCM-resistant plastic, and matched as closely as possible for absorbance at 650 nm (A_{650}) filled with 2 mL milliQ purified water (MQ water). Place tubes in the front cuvette holder with the diamond mark facing forward to further reduce variations.
3. Glass reagent bottles with plastic-lined screw caps, glass pipettes, and glass or plastic syringes with steel needles.
4. Dedicated plastic weighing boat (the larger kind) known to be free of detergents and well washed between uses.

General Precautions:

1. Wash your hands to remove natural skin lipids (fats and surfactants) and cosmetics containing detergents and conditioners (surfactants).
2. All labware coming in contact with any solid or liquid associated with the assay procedure must be free of traces of detergents, greases and organic solvents. Initially used glassware or plasticware should be thoroughly scrubbed with lab detergent in hot tap water, followed by copious hot tap water rinses, and three sequential, small volume ethanol rinses. Allow to drain and completely dry on paper towels. Traces of ethanol cause MB to become more soluble in DCM. This will give spuriously high A_{650} in reagent blanks and samples. If glassware is needed immediately, finish rinses with three sequential, small MQ water rinses, and shake out water droplets or drain briefly on paper towels.
3. To avoid cross-contamination, dedicate pipettes and droppers to the solutions they are used for, and if necessary add reagents to assay tubes without touching the inner walls of the tubes. Labeling dropper bulbs and containers-in-use keeps track of things.
4. Use a waste beaker for DCM extracts, and collect in labeled waste jug each day.

Preparation of reagents and how to use them:

1. MilliQ purified water: Use one liter glass bottles with plastic-lined screw caps. Follow posted operating procedure, using sterile technique* (fill bottle directly from white plastic outlet with small plastic hood in place, and replace blue cap when done). If refilling old MQ water bottles, do three sequential, small volume rinses before collecting a full bottle. Date and initial bottle.
* Examples of sterile technique: Never touch inside of container caps; keep containers tightly closed except when briefly pouring out contents; disinfect glassware with detergent – hot water – ethanol wash, dry outside surfaces with paper towels, and place upside down to drain and completely dry on clean paper towels inside large glass beakers.

2. Strong acid - ethanol glassware rinse: Place approximately 50 mL of concentrated fuming HCl (12 M, goggles in hood!) in a plastic squirt bottle, and fill to the 500 mL line with 95% or 100% ethanol (EtOH). This 1.2 M HCl-EtOH will remove MB from most glassware. Use sparingly once, followed by copious hot tap water to remove the acid, and three sequential, small EtOH rinses to remove the tap water, stray detergents and greases. Drain and completely dry on paper towels in test tube racks or glass beakers.

3. 0.01 M boric acid diluent for MB reagent: Place 0.62 g of boric acid (H_3B0_3, 61.8 g/mol) into a one liter graduated cylinder, fill half way with MQ water, and magnetically stir to wet the powder and dissolve it. Fill to mark, invert with parafilm cap to mix, place in 1 L bottle.

4. Stock 1 mg/mL methylene blue (MB) reagent: Weigh exactly 100 mg (to nearest 0.1 mg) of MB in the plastic boat. With a steel spatula, carefully transfer the MB powder to the boat. MB is a very mobile and finely divided black powder. Place notebook paper by the balance to capture MB particles, and use the small brush to clean the balance if necessary. Wash the paper off in the sink to avoid MB particles escaping into the lab area. Quantitatively transfer the MB powder into a 100 mL volumetric flask with a funnel by carefully adding a small volume of the boric acid diluent to the MB powder and stirring with a steel spatula. The MB powder is hard to wet and prone to "puffing" out of the weighing boat. Repeat the diluent-transfer step until there is no further blue residues in the boat (up to a

dozen times). Fill the flask to the 100 mL mark using a glass dropper for the last few milliliters. Cap with a ground-glass cap and invert the flask until all solid dissolves.

5. Working 0.1 mg/mL MB reagent: Place 10 mL of 1 mg/mL MB in a 100 mL graduated cylinder, add boric acid diluent to the mark, seal with parafilm and invert cylinder to mix. Pour into a capped bottle fitted with a pipette holder (2-5 mL disposable plastic pipette with top cut off and taped to bottle).

6. Acidic back-wash solution*: Heat is generated when concentrated sulfuric acid (18 M H_2SO_4) is diluted into water. Sulfuric acid in any dilution will destroy clothing. Wear goggles and do this in the fume hood. Use a bulb on a 10 mL glass pipette to draw up the concentrated sulfuric to the 3 mL mark. Slowly!, with swirling add 6.8 mL of concentrated sulfuric acid to 500 mL MQ water in a one liter volumetric flask. Add 50 g of monobasic sodium phospate monohydrate ($NaH_2PO_4 \bullet H_2O$, g/mol), swirl to dissolve, and bring to the 1000 mL mark.
* The sulfuric acid back-wash lowers blank A_{650} values and false-positive interferences. This may be needed for samples containing less than 0.1 ppm anionic surfactants.

7. Dichloromethane (DCM) extraction solvent: Place reagent grade DCM in a 100 mL glass bottle with a ground-glass stopper. Because DCM (boiling point °C) easily evaporates, use a 10 mL glass syringe with steel needle to remove assay portions. Pull syringe plunge gently to avoid DCM boiling at reduced pressure inside syringe. Because DCM will extract grease from your hands, occasionally check syringe for non-volatile residues by pulling in and expelling air until dry. Remove residues by pulling/expelling three small, sequential portions of DCM.

8. Sodium dodecyl sulfate (SDS) standards: Use reagent grade "sodium lauryl (docecyl) sulfate" powder, taken once from a large bottle and kept in a screw-capped 20 mL vial for use. Make a 1000 parts-per-million stock solution by accurately weighing 1.00 grams of SDS into a one liter volumetric flask, filling to the mark with MQ water, capping with a ground-glass stopper, and inverting until solids dissolve. From the 1000

ppm stock prepare a 1:100 dilution in MQ water (10.0 mL in 1 L volumetric flask) providing a 10 ppm SDS stock. Using six 100 mL volumetric flasks, make these working standards:
With a 10 mL glass pipette:
10.0 mL of 10 ppm to 100 mL = 1.0 ppm SDS
5.0 mL of " " to " " = 0.5 ppm SDS
2.5 mL of " " to " " = 0.25 ppm SDS,
and then with well-rinsed pipette:
10 mL of 1 ppm to " " = 0.10 ppm SDS
5.0 mL of " " to " " = 0.05 ppm SDS
2.5 mL of " " to " " = 0.025 ppm SDS
Like all surfactants, SDS will adhere to any surface, most certainly glass. Over time the more dilute SDS standards may lose proportionately more to surface adherence and possible degradation. Air and bacterial degradation is slowed in the refrigerator. Formaldehyde at 1-5% is used as a disinfectant to preserve environmental samples up to two weeks before analysis (three literature reports using 1%, 2.5%, and 5% formaldehyde).

<u>MBAS assay calibration procedure:</u>

1. In a test tube rack, line up and label eight 13 X 100 tubes, with clean screw caps in front of each: SB (solvent blank), RB (reagent blank), and .025, .05, .1, .25, .5, 1 ppm of SDS standard.
2. Into the SB tube pipette 2.1 mL MQ water, and into the RB tube 2.0 mL MQ water.
3. Into the rest of the tubes, from low to high ppm to avoid contamination, transfer 2.0 mL of each SDS standard with a 2 mL plastic syringe with steel needle, thoroughly pre-rinsed if previously used for standards . Deliver the solutions down the inside of the tubes to avoid losses at the upper rims.
4. Into all tubes except the SB tube add 0.10 mL of the 0.1 mg/mL MB reagent. Use the dedicated 0.5 mL glass pipette filled above the zero mark, adjusted with finger pressure to zero, then sequentially emptied in 0.1 mL aliquots (equal volumes) into five tubes. Refill to the 0.3 mL mark and deliver into last two tubes. Blow pipette into waste beaker and store in side holder on reagent bottle.
5. Add 2.0 mL of DCM to each tube and immediately cap tightly to avoid evaporation of DCM.

6. Holding each tube with finger tips on the cap, gently vortex for approximately 10 seconds. The contents should swirl up to the diamond mark, with a vortex down to the bottom of the tube.
7. Place the eight tubes in a standard bench-top, fixed angle, 12-place centrifuge, with pairs in opposing positions (balanced to avoid vibration), and turn lever to full speed for approximately one minute. This removes water droplets from the inside walls of the tubes, providing a stable A_{650} reading*.
   * If water is still a problem, remove the upper aqueous layer and add a small portion of sodium sulfate to dehydrate the DCM. Alternatively, trap water by passing the DCM layer through a DCM pre-rinsed plug of cotton or glass wool in a glass dropper, directly into the spectrophotometer cuvette.
8. Immediately read capped tubes in the front cuvette holder of the spectrophotometer, pre-zeroed in air (lid closed).

Results of first calibration run 7/29/08 by J.B.

Assay tube, with lower DCM phase in light beam.	A_{650} versus air. Two readings about 5 minutes apart.	Comments
SB (solvent blank)	.154, .159	Typical readings, can vary with tube position
RB (reagent blank)	.253, .257	Absorbance apparently due to MB impurities extracted into DCM.
0.025 ppm SDS	.262, .264	Barely above reagent blank; shows how accurate MB reagent delivery must be.
0.050 ppm SDS	.271, .269	
0.10 ppm SDS	.283, .287	
0.25 ppm SDS	.345, .336	Largest variation yet looks good on graph.
0.50 ppm SDS	.430, .431	
1.0 ppm SDS	.684, .685	Would be close to linear calibration if this one had been 0.61 A_{650}!

Assaying samples: Always run the reagent blank and at least one standard with your samples. Not knowing the anionic surfactant content of your samples (most will probably be in the ppb (parts-per-billion) range and therefore "zero" in our assay, start with just the 0.l ppm SDS standard in duplicate. Generally try to "bracket" your sample with a low and high standard once you have an idea of where your sample lies.

Limits of our MBAS assay at this time: Any results below 0.1 ppm are too close to the reagent blank values to be significant, given the variability of approximately + or – 0.03 A_{650} across a number of the screw-capped tubes used. For less than 0.1 ppm samples, transfer the DCM layer into a narrow (1.5 mL) glass cuvette to eliminate this variability. However, any water transferred will cloud the cuvette windows, leading to unstable readings.

Using the screw-capped assay tubes for educational purposes: Evaporation of DCM is prevented and the two-phase system is stable for at least one day. Increasing blue color of the lower DCM layer is readily apparent at 1 ppm or higher anionic surfactant. Digital photographs as "color comparators" can be used in public demonstrations and in slide presentations, similar to the Hellige, Inc., MBAS glass color comparator disk.

Further method optimization:
1. Reduce leakage of the screw-capped vials with an added liner-insert. Try the white Teflon disks used in HPLC autosampler vial caps.
2. Hannah will report on her methods of reducing the cloudy water droplets in the DCM phase, improving clarity of the DCM and stability of the spectrophometer readings.

Literature resources:
1. EPA-approved Method 5540C in "Standard Methods for the Examination of Water and Wastewater," (2000) is generally followed, substantially scaled down and simplified to a single-extraction method for student research and educational use. Sulfuric acid is replaced where possible with safer reagents such as boric acid. Dichloromethane (DCM) replaces more toxic chloroform.

2. USGS MBAS method 0-3128-95 (EPA 425.1, storet # 38260), in USGS open-file report #95-189 (1995). More detail and quality control of Method 5540C is documented.

To rationalize and optimize the chemistry of our single-extraction MBAS method, research literature back to the early 1950's on development of the method was critically reviewed:

3. Jurado, E., et.al, Chemosphere 65, 278 (2006) use Abbott's tetraborate buffer in a single-extraction method, and replace sulfuric acid with boric acid in the methylene blue reagent.
4. Koga, M., et.al., Analytical Sciences (Japan Soc. for Anal. Chem.) 15, 563 (1999), show that a 2:1 ratio of methylene blue to LAS is sufficient.
5. Wang, L. K., et.al., Ind. Eng. Chem. Prod. Res. Dev., 17 (3), 186 (1978), single-extraction methods compared and chloride interference quantitated.
6. Wang, L. K., et. al., Anal. Chem. 47(8), 1472 (1975), use azure A (demethylated methylene blue) to complex linear alkyl sulfonates (LAS), then titrate complex with a cationic dye to a methyl orange visual endpoint. There is no need for spectrophotometer and one can assay seawater, but this method is only good down to 3 ppm LAS.
7. Abbott, D. C., Analyst 87, 286 (1962) defines and pre-extracts methylene blue impurities with alkaline borate buffer.
8. Webster, H. L., et.al., Analyst 84, 552 (1959), use 1.2 M hydrochloric acid in ethanol to remove methylene blue from glassware.
9. Mukerjee, P., Anal. Chem. 28(5), 870 (1956). Original single-extraction method; calculated a maximum 0.2 ppm loss of LAS on the glass surface of a 100 mL volumetric flask.
10. Longwell, J., et.al., Analyst, 80, 167 (1955), original British MBAS method.
11. Edwards, G. R., et.al., Analyst 77, 205 (1952), original use of sulfuric acid and salt in the methylene blue reagent to decrease the solubility of the methylene blue-LAS complex in water.